IMMOBILIZATION OF α-AMYLASE (BAN) FOR SAGO STARCH HYDROLYSIS

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A report submitted in partial fulfillment
Of the requirements for the award
Of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical Engineering & Natural Resources
University College of Engineering & Technology Malaysia

NOVEMBER 2006

DECLARATION

I declare that this thesis entitled "Immobilization of α-Amylase (BAN) for sago starch hydrolysis" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....

Name : KHAIRIL NAZUAN BIN MOHD

Date : 20 November 2006

Dedicated to my beloved father, mother, and family……..

ACKNOWLEDGEMENT

In order to complete this research, I was in contact with many peoples, researchers, academicians and practitioners. All of them have assisted me in many ways towards completing this research. They also have contributed towards my understanding and thoughts. I would like to express my sincere appreciation to my supervisor, Mr Lau Sing Hui dan Miss Nina Suhaity Binti Azmi for their encouragement, guidance, critics and friendship in finishing my research.

I also would like to thanks the personnel of Faculty of Chemical Engineering and Natural Resource (FKKSA), especially lectures for their assistance and cooperation. Not forgotten to Mr. Joharizal Johari for their advices, motivation and ideas. Without their continued support and interest, this research would not have been the same as presented here.

My biggest thanks to the staff of FKKSA Chemical Laboratory especially Madam Norlia Mohammad, Miss Idayu and Mr. Anuar Ramli for their directly or indirectly influential and supportive in finishing this research.

My sincere appreciation also extends to all my colleagues, especially Mohd Rosli Ramly and others who have provided assistance at various occasions. Their views and tips are useful indeed. Your kindness is really appreciate and always in my mind forever.

Finally, my special appreciation is dedicated to my parents, Mr. Mohd bin Omar dan Madam Zainun binti Ismail and also my family for their tireless effort and endless moral support. Thank you.

ABSTRACT

α-Amylase produced from *Baccilus Amyliquifaciens* (termamyl) was immobilized by entrapment in calcium alginate gel capsules and it was used repeatedly in batch processes of starch hydrolysis. The degree of starch degradation and operational stability of the immobilized system were increased by tailoring the characteristics of the capsules. Capsules prepared from 2% (w/v) sodium alginate and 5% (w/v) CaCl2 were suitable for up to 20 repeated uses, losing only 30% of their initial efficiency. These alginate/silica capsules carrying α-Amylase retained 90% of their initial efficiency after 20 starch hydrolysis batches and released more than 10,700 mg of reducing sugars during a processing period of 160 h.

ABSTRACT

α-Amylase daripada *Baccilus Amyliquifaciens* (termamyl) dipegunkan dengan cara pemerangkapan di dalam kapsul kalsium alginat dan digunakan berulang-kali dalam proses hidrolisis kanji. Darjah penurunan kanji dan kestabilan operasi dalam sistem hidrolisis ini telah dapat ditingkatkan dengan menala karekteristik kapsul tersebut. Kapsul yang disediakan dengan menggunakan 2% (w/v) sodium alginat dan 5% (w/v) $CaCl_2$ adalah sangat sesuai digunakan sehingga 20 kali dengan hanya kehilangan 30% daripada kecekapannya pada permulaan proses. Kapsul alginat ini yang mengandungi α-Amylase dapat mencapai 90% daripada kecekapan permulaannya selepas 20 kali proses hidrolisis kanji dilakukan dan dapat membebaskan lebih daripada 10,700 mg gula penurun sepanjang tempoh proses selama 160 jam.

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LIST OF ABBREVIATION

BAN	α-Amylase
pH	A measure of acidity
UGI	A multi-component reaction in organic chemistry
MTT	A laboratory test and a standard colorimetric assay
UV	Ultraviolet
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD^+

CHAPTER 1

INTRODUCTION

1.1 Introduction

Enzymes are protein molecules which serve to accelerate the chemical reactions of living cells (often by several orders of magnitude). Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means unable to move or stationary. And that is exactly what an immobilized enzyme is: an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product.

Enzymes can denature due to solvent effects and mechanical shear forces. Recovery of enzymes from reaction solutions and separation of the enzymes from substrates and products are in general very difficult. These problems can be successfully tackled by immobilization of the enzyme.

The main advantages of immobilized enzymes are:

- Easy separation from reaction mixture, providing the ability to control reaction times and minimize the enzymes lost in the product.

- Re-use of enzymes for many reaction cycles, lowering the total production cost of enzyme mediated reactions.

- Ability of enzymes to replace multiple standard chemical steps and provide enatomerically pure products.

1.2 Problem Statement

Enzymes are protein molecules which serve to accelerate the chemical reactions of living cells (often by several orders of magnitude). Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means unable to move or stationary. And that is exactly what an immobilized enzyme is: an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product

1.3 Objective

The objective of this research is to study the immobilization of α-amylase (BAN) and in alginate beads for sago starch hydrolysis.

1.4 Scope

1 To study the immobilization of α-amylase and amyloglucosidase for sago starch hydrolysis
2 To investigate the relationship between bead size and alginate concentration with alginate capsules
3 To optimize the capsule's characteristic.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The hydrolysis of starch to products with low molecular weight, catalyzed by α-amylases is one of the most important commercial enzyme processes. The hydrolyzed products are widely applied in food, paper and textile industries. Industrial development of enzymic reactors requires the use of immobilized enzymes in order to reduce the cost of the biocatalyst. To a large extent this procedure prevents enzyme losses due to washout and at the same time maintains biocatalyst at high concentrations. Effective enzyme immobilization can be achieved using several techniques, one of which is encapsulation within a gel matrix. This immobilization technique consists of enclosing the enzyme in an aqueous solution inside a semipermeable membrane capsule. Basically, there are two main advantages of this immobilization method, the particle structure allows contact between the substrate and enzyme to be achieved and in addition it is possible to immobilize several enzymes at the same time .Encapsulation in Ca-alginate gels occurs under very mild conditions and is characterized by low cost and ease of use Moreover, by changing the gelation conditions it is possible to control easily some of the capsule characteristics, such as thickness or permeability to different substrates of the gel membrane

2.2 Method of immobilization

When immobilizing an enzyme to a surface, it is most important to choose a method of attachment that will prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. In other words, attach the enzyme but do as little damage as possible. Considerable knowledge of the active site of the enzyme will prove helpful in achieving this task. It is desired to avoid reaction with the essential binding site group of the enzyme. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity. In some cases, this protective function can be fulfilled by a substrate or a competitive inhibitor of the enzyme.

The surface on which the enzyme is immobilized is responsible for retaining the structure in the enzyme through hydrogen bonding or the formation of electron transition complexes. These links will prevent vibration of the enzyme and thus increase thermal stability. The micro environment of surface and enzyme has a charged nature that can cause a shift in the optimum pH of the enzyme of up to 2 pH units. This may be accompanied by a general broadening of the pH region in which the enzyme can work effectively, allowing enzymes that normally do not have similar pH regions to work together (Bentley *et al*., 1996).

2.2.1 Carrier-binding

The carrier-binding method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. The following picture shows how the enzyme is bound to the carrier:

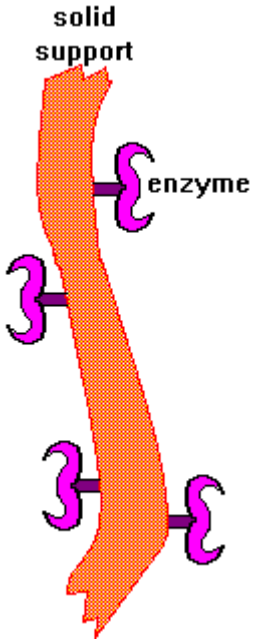

Figure 2.2.1 Carrier binding

The selection of the carrier depends on the nature of the enzyme itself, as well as the:

- Particle size
- Surface area
- Molar ratio of hydrophilic to hydrophobic groups
- Chemical composition

In general, an increase in the ratio of hydrophilic groups and in the concentration of bound enzymes, results in a higher activity of the immobilized enzymes. Some of the most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel.

According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified into:

- Physical Adsorption
- Ionic Binding
- Covalent Binding

2.2.1.1 Physical adsorption

This method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme or destruction of its active center. If a suitable carrier is found, this method can be both simple and cheap. However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during use due to a weak binding force between the enzyme and the carrier. The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-furanosidase onto aluminum hydroxide. The processes available for physical adsorption of enzymes are:

- Static Procedure
- Electro-deposition
- Reactor Loading Process
- Mixing or Shaking Bath Loading

Of the four techniques, the most frequently used in the lab is *Mixing-Bath Loading.* For commercial purposes the preferred method is *Reactor Loading.*

A major advantage of adsorption as a general method of immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. Adsorption tends to be less disruptive to the enzymatic protein than chemical means of attachment because the binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. In this respect, the method bears the greatest similarity to the situation found in natural biological membranes and has been used to model such systems.

Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Another disadvantage is non-specific, further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate.

Adsorption of the enzyme may be necessary to facilitate the covalent reactions described later in this presentation. Stabilization of enzymes temporarily adsorbed onto a matrix has been achieved by cross-linking the protein in a chemical reaction subsequent to its physical adsorption (Bentley *et al*., 1996).

2.2.1.2 Ionic bonding

The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues.

Polysaccharides and *synthetic polymers* having ion-exchange centers are usually used as carriers. The binding of an enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme. Therefore, this method yields immobilized enzymes with high activity in most cases.

Leakage of enzymes from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. This is because the binding forces between enzyme proteins and carriers are weaker than those in covalent binding.

The main difference between ionic binding and physical adsorption is that the enzymes to carrier linkages are much stronger for ionic binding although weaker than in covalent binding (Bentley *et al*., 1996)..

2.2.1.3 Covalent binding

The most intensely studied of the immobilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics: (1) the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and (2) the active site of the enzyme must be unaffected by the reagents used.

The covalent binding method is based on the binding of enzymes and water-insoluble carriers by covalent bonds. The functional groups that may take part in this binding are listed below:

- Amino group
- Carboxyl group
- Sulfhydryl group,
- Hydroxyl group
- Imidazole group
- Phenolic group
- Thiol group
- Threonine group
- Indole group

This method can be further classified into diazo, peptide and alkylation methods according to the mode of linkage. The conditions for immobilization by covalent binding are much more complicated and less mild than in the cases of physical adsorption and ionic binding. Therefore, covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of

activity and/or changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength.

Covalent attachment to a support matrix must involve only functional groups of the enzyme that are not essential for catalytic action. Higher activities result from prevention of inactivation reactions with amino acid residues of the active sites. A number of protective methods have been devised:

- Covalent attachment of the enzyme in the presence of a competitive inhibitor or substrate.
- A reversible, covalently linked enzyme-inhibitor complex.
- A chemically modified soluble enzyme whose covalent linkage to the matrix is achieved by newly incorporated residues.
- A zymogen precursor.

Hence, covalent binding can be brought about by the following:

- Diazotization :
- Amide bond formation :
- Alkylation and Arylation:
- Schiff's base formation :
- Amidation reaction :
- Thiol-Disulfide interchange :
- UGI reaction
- Mercury-Enzyme interchange
- Gamma-Irradiation induced coupling
- Carrier binding with bifunctional reagents :

The active site of the enzyme must not be hindered. There must be ample space between the enzyme and the backbone.

It is possible in some cases to increase the number of reactive residues of an enzyme in order to increase the yield of the immobilized enzyme. This provides alternative reaction sites to those essential for enzymatic activity. As with cross-linking, covalent bonding should provide stable, immobilized enzyme derivatives that do not leach enzyme into the surrounding solution. The wide variety of binding reactions and insoluble carriers (with functional groups capable of covalent coupling or being activated to give such groups) makes this a generally applicable method of immobilization. This is true even if very little is known about the protein structure or active site of the enzyme to be coupled (Bentley *et al*., 1996).

2.2.2 Cross linking

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix.. Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support. This will result in relatively low enzymatic activity. Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage from polyacrylamide gels.

Since the enzyme is covalently linked to the support matrix, very little desorption is likely using this method. Marshall (1973), for example, reported that carbamy phosphokinase cross-linked to alkyl amine glass with glutaraldehyde lost only 16% of its activity after continuous use in a column at room temperature for fourteen days.

The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These harsh

conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity (Bentley *et al*., 1996).

2.2.3 Entrapping

The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It is done in such a way as to retain protein while allowing penetration of substrate. It can be classified into lattice and microcapsule types.

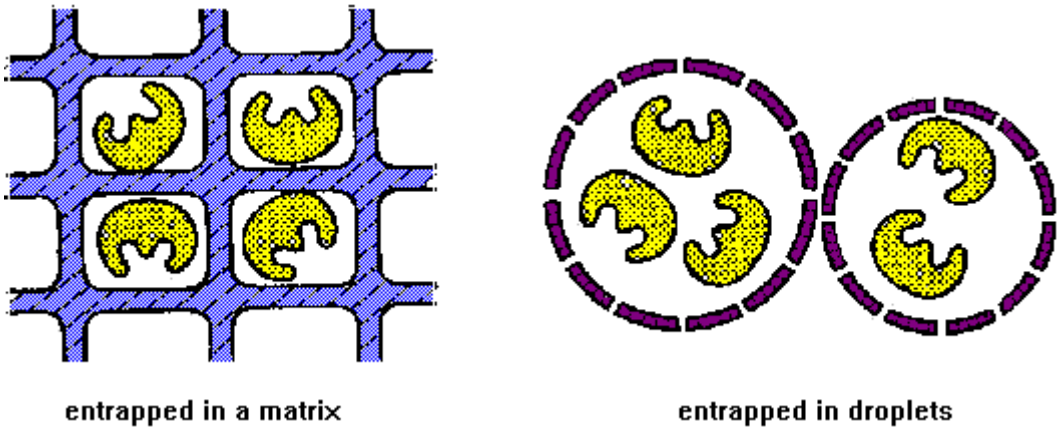

Figure 2.2.3 Entrapping

This method differs from the covalent binding and cross linking in that the enzyme itself does not bind to the gel matrix or membrane. This results in a wide applicability. The conditions used in the chemical polymerization reaction are relatively severe and result in the loss of enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required.

Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as *polyarylamide, polyvinylalcohol, etc...* and natural polymer (starch) have been used to immobilize enzymes using this technique.

Microcapsule-Type entrapping involves enclosing the enzymes within semi permeable polymer membranes. The preparation of enzyme micro capsules requires extremely well-controlled conditions and the procedures for micro capsulation of enzymes can be classified as:

- Interfacial Polymerization Method: In this procedure, enzymes are enclosed in semi permeable membranes of polymers. An aqueous mixture of the enzyme and hydrophilic monomer are emulsified in a water-immiscible organic solvent. Then the same hydrophilic monomer is added to the organic solvent by stirring. Polymerization of the monomers then occurs at the interface between the aqueous and organic solvent phases in the emulsion. The result is that the enzyme in the aqueous phase is enclosed in a membrane of polymer.

- Liquid Drying: In this process, a polymer is dissolved in a water-immiscible organic solvent which has a boiling point lower than that of water. An aqueous solution of enzyme is dispersed in the organic phase to form a first emulsion of water-in-oil type. The first emulsion containing aqueous micro droplets is then dispersed in an aqueous phase containing protective colloidal substances such as gelatin, and surfactants, and a secondary emulsion is prepared. The organic solvent in then removed by warming in vacuum. A polymer membrane is thus produced to give enzyme micro capsules.

- Phase Separation: One purification method for polymers involves dissolving the polymer in an organic solvent and re-precipitating it. This is accomplished by adding another organic solvent which is miscible with the first, but which does not dissolve the polymer.

The form an of immobilized enzyme can be classified into four types: particles, membranes, tubes, and filters. Most immobilized enzymes are in particle form for ease of handling and ease of application.

- Particles - The particle form is described in the above section.
- Membranes - Enzyme membranes can be prepared by attaching enzymes to membrane-type carriers, or by molding into membrane form. The molding is done after the enzymes have been enclosed within semi-permeate membranes of polymer by entrapment.

- Tubes - Enzyme tubes are produced using Nylon and polyacrylamide tubes as carriers. The polymer tube is first treated in a series of chemical reactions and the enzyme is bound by diazo coupling to give a tube in a final step.

- Fibers - Enzymes that have been immobilized by entrapment in fibers to form enzyme fibers.

The solid supports used for enzyme immobilization can be *inorganic* or *organic*. Some organic supports include: Polysaccharides, Proteins, Carbon, Polystyrenes, Polyacrylates, Maleic Anhydride based Copolymers, Polypeptides, Vinyl and Allyl Polymers, and Polyamides (Bentley *et al*., 1996).

2.3 Properties of Immobilized Enzymes

It is important to understand the changes in physical and chemical properties which an enzyme would be expected to undergo upon insolubilization if the best use is to be made of the various insolubilization techniques available. Changes have been observed in the stability of enzymes and in their kinetic properties because of the microenvironment imposed upon them by the supporting matrix and by the products of their own action.

2.3.1 Stability

The stability of the enzymes might be expected to either increase or decrease on insolubilization, depending on whether the carrier provides a microenvironment capable of denaturing the enzymic protein or of stabilizing it. Inactivation due to autodigestion of proteolytic enzymes should be reduced by isolating the enzyme molecules from mutual attack by immobilizing them on a matrix. It has been found that enzymes coupled to inorganic carriers were generally more stable than those attached to organic polymers when stored at 4 or 23 ° centigrade. Stability to denaturing agents may also be changed upon insolubilization (Godia *et al*., 1995)

2.3.2 Kinetic Properties

Changes in activity of enzymes due to the actual process of insolubilization have not been studied very much. There is usually a decrease in specific activity of an enzyme upon insolubilization, and this can be attributed to denaturation of the enzymic protein caused by the coupling process. Once an enzyme has been insolubilized, however, it finds itself in a microenvironment that may be drastically different from that existing in free solution. The new microenvironment may be a result of the physical and chemical character of the support matrix alone, or it may result from interactions of the matrix with substrates or products involved in the enzymatic reaction.

The Michaelis constant has been found to decrease by more than one order of magnitude when substrate of opposite charge to the carrier matrix was used. Again, this only happened at low ionic strengths, and when neutral substrates were used. The electrostatic potential was calculated by insertion of the Maxwell-Bottzmann distribution into the Michaelis-Menton equation using the changes in Michaelis constant, and good agreement was obtained with the value for the electrostatic potential calculated from the pH-activity shifts.

The diffusion of substrate from the bulk solution to the micro-environment of an immobilized enzyme can limit the rate of the enzyme reaction. The rate at which substrate passes over the insoluble particle affects the thickness of the diffusion film, which in turn determines the concentration of substrate in the vicinity of the enzyme and hence the rate of reaction.

The effect of the molecular weight of the substrate can also be large. Diffusion of large molecules will obviously be limited by steric interactions with the matrix, and this is reflected in the fact that the relative activity of bound enzymes towards high molecular weight substrates has been generally found to be lower than towards low molecular weight substrates. This, however, may be an advantage in some cases, since the immobilized enzymes may be protected from attack by large inhibitor molecules (Godia *et al*., 1995).

2.4 Hydrolysis Process

Hydrolysis is a chemical reaction or process in which a molecule is split into two parts by reacting with a molecule of water, which has the chemical formula H_2O. One of the parts gets an OH^- from the water molecule and the other part gets an H^+ from the water (http://en.wikipedia.org/wiki/Hydrolysis). It is one of the mechanisms for the breakdown of food by the body, as in the conversion of starch to glucose.

There are two types of hydrolysis, acid and enzymatic. Feedstocks that may be appropriate for acid or enzymatic hydrolysis typically are plant-based materials containing cellulose. These include forest material and sawmill residue, agricultural residue, urban waste, and waste paper

.

All plants have structural components composed of lignocellulosic fibers, which in turn are comprised of three major fractions: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are chains of sugar molecules that can be broken down chemically or biologically into the component sugars. The sugars are then

fermented using yeast or bacteria to produce ethanol, which is then distilled to a higher concentration for final use.

Sugars can also be converted to levulinic acid and citric acid. Levulinic acid is a versatile chemical that is a precursor to other specialty chemicals, fuels and fuels additives, herbicides, and pesticides. The largest application for citric acid is in the beverage industry, which accounts for about 45 percent of the market for this product. Citric acid is also used in a wide variety of candies, frozen foods, and processed cheeses and as a preservative in canned goods, meats, jellies, and preserves.

Lignin binds cellulose and hemicellulose together and cannot be broken down to form sugars. At this point, the most cost-effective use for lignins is as a fuel for biomass-to-energy facilities.

2.5 α-Amylase(BAN)

Alpha amylase is classified as family 13 of the glycosyl hydrolases. The structure is an 8 stranded alpha/beta barrel containing the active site, interrupted by a ~70 a.a. calcium-binding domain protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal Greek key beta-barrel domain.

O-Glycosyl hydrolases (EC 3.2.1.-) are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Dziezak, J.D, 1991).

2.6 Sago Starch

Sago is a powdery starch made from the processed pith found inside the trunks of the Sago Palm *Metroxylon sagu*. Sago forms a major staple food for the lowland peoples of New Guinea and the Moluccas.

Processed starch known as sago is also made from some cycad plants, and is a less frequent food source for some peoples of the Pacific and Indian Oceans. There is a large difference both biologically and dietarily between the two types of sago. Sago as a major dietary food source comes mainly from a palm in the genus *Metroxylon*. Despite their common name, cycads are not palms (i.e. they are not members of the family Arecaceae but rather from Cycadaceae, a vastly different taxonomic order: cycads are gymnosperms, while palms are angiosperms).

Because sago flour made from *Metroxylon* is the most widely used form, this article discusses sago from *Metroxylon* unless otherwise specified.

Sago flour (*Metroxylon*) is nearly pure carbohydrate and has very little protein, vitamins, or minerals. However, as sago palms are typically found in areas unsuited for agriculture, sago cultivation is often the most ecologically appropriate form of land-use, and the nutritional deficiencies of the food can often be compensated for with other readily available dietary items.

One hundred grams of dry sago yields 355 calories, including an average of 94 grams of carbohydrate, 0.2 grams of protein, 0.5 grams of dietary fiber, 10mg of calcium, 1.2mg of iron, and negligible amounts of fat, carotene, thiamine, and ascorbic acid.

Sago can be stored for weeks or months, although generally it is eaten quickly after it is processed (Isa *et al*., 1999)

2.7 Enzyme assay

Enzyme assays are laboratory methods for measuring enzymatic activity. They are vital for the study of enzyme kinetics and enzyme inhibition. Amounts of enzymes can either be expressed as molar amounts, as with any other chemical, or measured in terms of activity.

Enzyme activity = moles converted per unit time = rate × reaction volume. Enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, which should be specified. The SI unit is the katal, 1 katal = 1 mol s^{-1}, but this is an excessively large unit. A more practical and commonly-used value is 1 enzyme unit (EU) = 1 μmol min^{-1} (μ = micro, x 10-6). 1 U corresponds to 16.67 nanokatals.

The specific activity of an enzyme is another common unit. This is the activity of an enzyme per milligram of total protein (expressed in μmol $min^{-1}mg^{-1}$). Specific activity gives a measurement of the purity of the enzyme.

2.8 Types of enzyme assay

All enzyme assays measure either the consumption of substrate or production of product over time. This gives the rate of reaction. A large number of different methods of measuring the concentrations of substrates and products exist and many enzymes can be assayed in several different ways.

In general, enzyme assays can be split into two groups continuous assays, where the assay gives a continuous reading of activity, and discontinuous assays, where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

2.8.1 Continuous assay

Continuous assays are most convenient, with one assay giving the rate of reaction with no further work necessary. There are many different types of continuous assays.

2.8.1.1 Spectrophotometric

In spectrophotometric assays, you follow the course of the reaction by measuring a change in how much light the assay solution absorbs. If this light is in the visible region you can actually see a change in the color of the assay, these are called colorimetric assays. The MTT assay, a redox assay using a tetrazolium dye as substrate is an example of a colorimetric assay.

UV light is often used, since the common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms. A oxidoreductase using NADH as a substrate could therefore be assayed by following the decrease in UV absorbance at 340 nm as it consumes the coenzyme.

2.8.1.2 Fluorimetric

Fluorescence is when a molecule emits light of one wavelength after absorbing light of a different wavelength. Fluorometric assays use a difference in the fluorescence of substrate from product to measure the enzyme reaction. These assays are in general much more sensitive than spectrophotometric assays, but can suffer from interference caused by impurities and the instability of many fluorescent compounds when exposed to light.

An example of these assays is again the use of the nucleotide coenzymes NADH and NADPH. Here, the reduced forms are fluorescent and the oxidized forms

non-fluorescent. Oxidation reactions can therefore be followed by a decrease in fluorescence and reduction reactions by an increase. Synthetic substrates that release a fluorescent dye in an enzyme-catalyzed reaction are also available, such as 4-methylumbelliferyl-β-D-glucuronide for assaying β-galactosidase.

2.8.1.3 Calorimetric

Calorimetry is the measurement of the heat released or absorbed by chemical reactions. These assays are very general, since many reactions involve some change in heat and with use of a microcalorimeter, not much enzyme or substrate is required. These assays can be used to measure reactions that are impossible to assay in any other way.

2.8.1.4 Chemiluminescencent

Chemiluminescence is the emission of light by a chemical reaction. Some enzyme reactions produce light and this can be measured to detect product formation. These types of assay can be extremely sensitive, since the light produced can be captured by photographic film over days or weeks, but can be hard to quantify, because not all the light released by a reaction will be detected.

The detection of horseradish peroxidase by enzymatic chemilumiescence (ECL) is a common method of detecting antibodies in western blotting. Another example is the enzyme luciferase, this is found in fireflies and naturally produces light from its substrate luciferin.

2.8.2 Discontinuous assay

Discontinuous assays are when samples are taken from an enzyme reaction at intervals and the amount of product production or substrate consumption is measured in these samples.

2.8.2.1 Radiometric

Radiometric assays measure the incorporation of radioactivity into substrates or its release from substrates. The radioactive isotopes most frequently used in these assays are ^{14}C, ^{32}P, ^{35}S and ^{125}I. Since radioactive isotopes can allow the specific labelling of a single atom of a substrate, these assays are both extremely sensitive and specific. They are frequently used in biochemistry and are often the only way of measuring a specific reaction in crude extracts (the complex mixtures of enzymes produced when you lyse cells).Radioactivity is usually measured in these procedures using a scintillation counter.

2.8.2.2Chromatographic

Chromatographic assays measure product formation by separating the reaction mixture into its components by chromatography. This is usually done by high-performance liquid chromatography (HPLC). Although this approach can need a lot of material, its sensitivity can be increased by labelling the substrates/products with a radioactive or fluorescent tag.

2.9 Factors to control in assays

2.9.1 Temperature

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. As shown in Figure 13, the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most animal enzymes rapidly become denatured at temperatures above 40·C, most enzyme determinations are carried out somewhat below that temperature.

Over a period of time, enzymes will be deactivated at even moderate temperatures. Storage of enzymes at 5·C or below is generally the most suitable. Some enzymes lose their activity when frozen.

2.9.2 Enzyme concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; i.e., the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. Graphically this can be represented as:

The amount of enzyme present in a reaction is measured by the activity it catalyzes. The relationship between activity and concentration is affected by many factors such as temperature, pH, etc. An enzyme assay must be designed so that the observed activity is proportional to the amount of enzyme present in order that the enzyme concentration is the only limiting factor. It is satisfied only when the reaction is zero order.

2.9.3 Substrate concentration (Michaelis-Menten kinetics)

Enzymes are not passive surfaces on which reactions take place but rather, are complex molecular machines that operate through a great diversity of chemical mechanisms. According to Michaelis-Menten kinetics, enzyme-substrate reactions are actually comprised of two elementary reactions. The first is the when the substrate forms a complex with the enzyme and then in the second, the complex decomposes to product and enzyme.

$$\text{Enzyme} + \text{Substrate} \underset{k-1}{\overset{k1}{<----->}} \text{Complex} \xrightarrow{k2} \text{Products} + \text{Enzyme}$$

According to this model, when the substrate concentration becomes high enough to entirely convert the entire enzyme to the complex form, the second step of the reaction becomes the rate-limiting step. Therefore, the overall conversion to product becomes insensitive to further increases in substrate concentration. The general expression for the rate of this reaction (velocity) becomes:

$$v = d[P]/dt = k2*[complex]$$